Role of Tumor Necrosis Factor – A (TNF – A) (G308A) Gene Polymorphism and Levels in Coronary Artery Patients in thi-Qar Province

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Abstract
The present study was carried out in the labs of Technical Institute –Al-Nasiriyah /Community Health Department and Nasiriyah Heart Center in Thi-Qar province, during the period of research was extended from January to July 2022. The aim of study was to determination polymorphisms of TNF-α gene in patients with Coronary Artery Disease and measuring its levels in serum using a technique enzyme-linked immune sorbent adsorptive (ELISA). The study included a total of 100 Iraqi patients with coronary artery disease (CAD) and healthy control group their age between 3-85 years. DNA has been isolated and PCR was performed by using primers specific for genotypes of TNF-α gene(308 A/G). The results showed that Gel Electrophoresis of PCR amplified TNFα-308 product with molecular size 107 bp. Frequency of genotypes for the TNF-α gene showed a high frequency of GG genotype compared to the AG and AA genotypes in all study groups, with no significant differences between cardiovascular patients and control group. The immunological study showed that there was a significant increase of concentrations of (TNF-α) in patients compared to the healthy control group. Thus it seems There is no correlation between TNF-α gene polymorphisms and the risk of coronary artery disease in Thi- Qar province and the Serological Assay (ELISA) showed a significant increase in the concentration of TNF-α in patients with coronary artery disease compared with the healthy control group.
Keywords: Coronary Artery Disease , Polymorphism , Tumor Necrosis Factor – α (TNF – α), Promoter regionof Tumor Necrosis Factor – α gene.

1. Introduction

Coronary artery disease (CAD) is a leading cause of death worldwide. It represents the manifestation of atherosclerosis in the coronary arteries, which supply the myocardium with oxygen and other nutrients(1). Atherosclerosis is an inflammatory condition of the arteries that has profound incidence and increasing prevalence . Endothelial cells (ECs) form the inner lining of blood vessels, acting as both a protective barrier and sensor for detecting changes in blood flow (2). The complex process of atherosclerosis begins early in life and is thought to initiate with dysfunction of endothelial cells that line the coronary arteries; these cells are no longer able to appropriately regulate vascular tone (narrowing or constriction of the vessels). There are several factors that contribute to coronary artery disease. However, these factors vary from one person to another. Genetics is considered to be one of the factors influencing the development of CAD. Some studies have reported 50 risk points in the human genome that can influence CAD development(3). Genotyping common single nucleotide polymorphisms (SNPs) within a potential CAD–related gene is an essential and efficient method to detect genetic risk markers(4). TNF-α is a strong proinflammatory and immunomodulatory cytokine that intervenes inflammatory diseases and is produced by activated macrophages (5). TNF- α is a T helper-1 cytokine produced by lymphocytes, macrophages and trophoblasts during pregnancy(6). TNF-α elicits the broadest spectrum of biological activities and has a major role in the cytokine network (7). TNF plays a vital role in the typical immune response through the regulation of a number of pathways encompassing an immediate inflammatory reaction with significant innate immune involvement as well as cellular activation with subsequent proliferation and programmed cell death or necrosis(8).

Many studies have examined the effect of cytokines polymorphisms such as IL-6 , IL-10 , IL-18 , TNF-a and interferon-Y and evaluate its influence on coronary artery disease (9, 10, 11, 12, 13, 14). Several types of immune cells, including macrophages, T and B lymphocytes also accumulate in the arterial walls and are involved in the development of atherosclerotic disorders through cytokines and other mediators(15). Genetic mutations are a form of genetic polymorphism. Genetic mutation is a permanent alteration within the polymer sequence that produces up a gene, specified that the sequence differs from what is found in majority of individuals (16, 17) .Mutations will have an effect on anywhere from one polymer (DNA) building block (base pair) to an oversized phase of a chromosome that features multiple genes (18, 19, 20).

2. Materials and Methods

Samples Collection

This study was performed on 100 Iraqi patients

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with coronary artery disease (CAD), who attended to Nasiriyah Heart Center and healthy control group. in the period from the January to July 2022. Blood samples were collected by venipuncture from 61 patients and 39 controls (Five milliliters of venous blood) were drawn by disposable syringe under aseptic technique. Each blood sample was divided into two parts (Three milliliters were put directly in a sterile tube containing EDTA for DNA extraction and two milliliters were placed in a sterile plane tube(without EDTA) and allowed to clot, then serum was separated by centrifugation at 4000 rpm for 15 minutes. The serum was stored at -20 ˚C freezing . These sera (61 patients and 28 controls) were used for estimating the concentration of Tumor Necrosis Factor –α (TNF-α) .

Molecular Study

DNA Extraction

DNA was extracted from blood samples according to the leaflet attached with DNA Extraction Kit (Geneaid /Thailand)
Which includes the following steps :

DNA Template and Polymerase Chain Reaction (PCR)

PCR technique was used to amplify the TNF-α gene according to (21) and used the following Primers for the PCR technique. Primers (forward and reverse) as the table (1) the kit provide by Solarbio science and tecnology company.

Table(1): Oligonucleotide Primer Sequences used for Amplification of TNF-α gene.			
Primers			Primer sequences
Primer ofTNFα	308G/A	F	5' AGGCAATAGGTTTTGAGGGCCAT -3'
		R	5' TCCTCCCTGCTCC GATTCCG -3'

Primers (F,R),D.W and DNA were mixed in master mix tube (20 µl)As showen in table(2)

Table (2): PCR reaction for amplification of TNF-α gene:	
Materials	Volume
Master Mix	25 µl
Primer Forward	0.5 µM
Primer Reverse	0.5 Mm
DNA	15 µl
D.W	-
Total	50 µl

The master mix tube put in the thermal cycle according to table (3). The primer pairs were included in the PCR for simultaneous amplification of fragments in TNF-α gene.

Table (3): PCR condition for amplification of TNF-α gene :				
No of steps	Steps	Temperature	Time	No . of cycle
1	Denaturation 1	94 ˚C	3 min	1
2	Denaturation 2	95 ˚C	30 sec	30
3	Annealing	60 ˚C	60 sec	
4	Extension 1	72 ˚C	60 sec	
5	Final Extension 2	72 ˚C	5 min	1

The products were transfered by the electrophoresis apparatus. The condition of electrophoresis was (1% agarose concentration), stained with ethidium Bromide (0.5µl/ml). under voltage 105 V and 400 mA for 40 minutes.

Product Analysis

The products were transfered by the electrophoresis apparatus by dissolving (1.2) of the Agarose gel in 60 mL of the TBE to become the final concentration (2%) and Visualization of amplified product was conducted by U.V transilluminator (302nm). The size of amplified product was determined according to ladder marker and imaged by digital camera.

Immunilogical Study

Determination of serum levels of TNF-α

The sera of 88 patients and controls were assessed for the level of TNF-α, by means of ELISA (technique enzyme-linked immune Sorbent adsorptive) kit are employing the quantitative sandwich that were based on similar principles according to the Bioassay Technology laboratory company.
Two milliliters were placed in a sterile plane tube and allowed to clot, then serum was separated by centrifugation at 4000 rpm for 15 minutes. The serum was stored at -20 C˚ freezing . These sera (61 patients and 27 healthy controls) were used for estimating The concentration of TNF-α.

3. Results

Molecular Studies

Gel Electrophoresis of PCR amplified TNF-α(-308 A/G)

The location of TNF-α(-308 A/G) (rs 1800629) polymorphisms in the TNF-α promoter and the electrophoresis results of PCR are shown in figure (1),

the gel Electrophoresis of PCR amplified TNF-α(-308 A/G) product with molecular size 107 bp. M: 100 bp molecular ladder. The condition of electrophoresis was (1% agarose concentration),stained with ethidium Bromide.

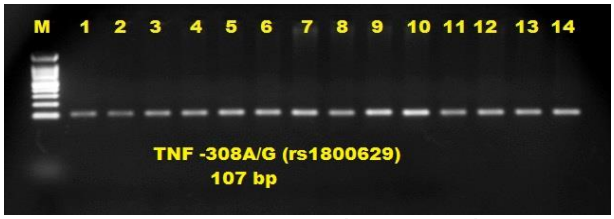

Fig.(1): Gel Electrophoresis of PCR amplified TNF-a(-308 A/G) product with molecular size 107bp.M:100 bp molecular ladder ,Lanes 1-14: positive amplification. The condition of electrophoresis were (1% agarose concentration), stained with ethidium Bromide (0.5 $\mu m/ml$ *).under voltage 105V and 400 mA for 40 minutes.*

Frequency of genotypes for the TNF-α gene samples of CAD patients and healthy controls

The TNF-α gene is located on the short arm of chromosome 6 within the major histocompatibility complex, as it is now known that genetic alterations at the TNF-α locus are directly involved in TNF-α production.

The frequencies of human TNF-α gene 308 A/G polymorphism in cardiovascular patients and control group are given in table (4).

The current study showed a high frequency of GG genotype compared to the AG and AA genotypes in all study groups, with no significant differences between cardiovascular patients and control group as show in table (4) .

Table (4): Distribution of genotypes for the TNF-α gene samples of CAD patients and healthy Control.

Alleles	No.(%)	
G	20 (100)	
Genotypes	Patients	Control
GG	10/10 (100%)	10/10(100%)
Odds ratio	1.00	
95 % CI:	0.2895 to 3.4543	

Immunological study

Serum TNF-α Concentration of healthy and CAD Patients

The results of this study showed there was a significant increase (P<0.05) of concentrations of (TNF-α) as seen in table (5) as the rate of concentration of TNF-α in patients (73.624 pg / ml) compared to the healthy control group (63.037 pg/ml) with a significant difference (0.009)

Table (5): Serum TNF-α, Concentration of healthy and CAD Patients :

Group Statistics							
Parameters	Group	N	Mean	Std. Deviation	T	Df	P. Value
TNF-α pg / ml	Patients	61	73.624	15.310	2.692	86	0.009
	Healthy	27	63.037	20.404			
(P<0.05) df : degree of freedom							

4. Discussion

Molecular Study

Cardiovascular disease is a complex genetic disease influenced by both genetic and environmental factors and characterized by high blood pressure, Age, gender, lifestyle (sedated life, obesity, excessive alcohol consumption, salty diet, stress) and metabolic diseases such as diabetes mellitus are important factors influencing the development of hypertension. In this study, we investigated the effects of polymorphism of TNFα-308 A/G and its possible effects on the etiology of patients with cardiovascular disease.

The functional role of the TNF-α G-308A polymorphism is currently unclear. However, it is located within a consensus sequence of the AP-2 transcription factor in the promoter region of the gene (22). The TNF α G-308A mutation in the promoter region of TNFα acts in vitro as a much stronger transcriptional activator than the wild-type of TNF α and it is suggested that a higher transcriptional activity would result in higher TNFα concentrations followed by decreased insulin sensitivity, Therefore, this variant could play a role in the development of obesity, which are risk factors for heart disease (23). Also, a study of (24), concluded the level of TNF α mR

NA in adipose tissue are significantly associated with the percentage of body fat in human obese subjects. Also, a study of reported a correlation between the TNF α locus and obesity in families of French-Canadian origin, has also found a significant association between TNF α locus and high blood pressure (25).The study by(26), reported that genes for proinflammatory cytokines such as IL-6 and TNF*α* are recognized as triggers of systemic and local manifestations of rheumatoid arthritis. The study by(27) showed the A allele of TNF*α* polymorphism −308G>A (rs1800629) has been associated with an increased risk of cardiovascular disease in rheumatoid arthritis patients who carry at least one copy of a common epitope.

Immunological Study

The present study investigated the concentration of TNF-α increases significantly compared with control group. The current study agreed with study of(28), their study showed a patient with CVD have high concentration of TNF-α . The study of(29), showed the TNF was increased significantly in CVD patients and their study concluded the CVD is associated with chronic, low-level inflammation, including elevations in circulating pro-inflammatory cytokines as IL-6 and IL-10 and acute phase reactant CRP. It is difficult to say whether inflammation is a cause or a consequence of certain disorders, but evidence suggests that it may play a major role in the pathogenesis of cardiovascular disease and other chronic diseases. In contrast the study of (30) showed the serum TNF-α levels were not affected in the

infective endocarditis (IE) process. Further understanding of the role of serum cytokine concentrations in the diagnosis, treatment, monitoring, and prognosis of IE may be valuable under the condition of suspected diagnosis, especially when pathogens cannot be detected in blood cultures. TNF-a is a key molecule in peripheral insulin resistance, and this resulte may be disagreed with the results of the current study in low TNF-α concentration in heart patients. In addition, TNF-a appears to be involved in the pathogenesis of atherosclerosis and heart failure. A marked increase in IL-6 and IL-10 is provoked by direct exercise and exertion anti-inflammatory effects by inhibiting TNF-a and stimulating IL-6 (31).

A study of (32), suggested an important issue related to the development of atherosclerosis in patients with rheumatoid arthritis is the search for the causes of endothelial dysfunction. In this context, proinflammatory cytokines such as TNF-α has been linked to carotid Intima-Media Thickness.

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